4.20 Reduced-risk insecticides in Neotropical stingless bee species: impact on survival and activity

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Abstract

Background**:** As honeybees are the main pollinator species subject to an intense research regarding effects of pesticides, other ecologically important native bee pollinators have received little attention in ecotoxicology and risk assessment of pesticides in general, and insecticides in particular, some of which are perceived as reduced-risk compounds. Here the impact of three reduced-risk insecticides – azadirachtin, spinosad, and chlorantraniliprole – was assessed in two species of stingless bees, *Partamona helleri* and *Scaptotrigona xanthotrica*, which are important native pollinators in Neotropical America. The neonicotinoid imidacloprid was used as a positive control.

Results: Spinosad exhibited high oral and contact toxicities in adult workers of both species at the recommended label rates, with median survival times (LT_{50}s) ranging from 1 to 4 h, whereas these estimates were below 15 min for imidacloprid. Azadirachtin and chlorantraniliprole exhibited low toxicity at the recommended label rates, with negligible mortality that did not allow LT_{50} estimation. Sublethal behavioral assessments of these two insecticides indicated that neither one of them affected the overall group activity of workers of the two species. However, both azadirachtin and chlorantraniliprole impaired individual flight take-off of *P. helleri* and *S. xanthotrica* worker bees, which may compromise foraging activity, potentially leading to reduced colony survival.

Conclusion: These findings challenge the common perception of non-target safety of reduced-risk insecticides and bioinsecticides, particularly regarding native pollinator species.

Keywords: behavioral impact; biopesticides; colony and individual level effects; native bee pollinators; sublethal effects.

1. IntroductionThe honeybee is perceived as very sensitive to insecticides compared to other arthropod species. [1-3] Therefore this species has for some time been the representative model pollinator because it is widely available globally and inexpensive to use as an environmental bioindicator of pesticide pollution. [3,4] However, a recent meta-analysis study provided support for such use of honeybees, a 10-fold sensitivity ratio correction seems necessary for the extrapolation of insecticide toxicity results from the honeybee to other bee species. [5] Such fact has obscured the importance of stingless bees and only little research has undertaken on this topic. [5-8]

Stingless bees species are the primary pollinators of wild and cultivated plants in Neotropical America [9-12] and they may be important even in the presence of the honeybee. [5,8,9] Therefore, stingless bees demand more attention regarding the potential effects of pesticides in this particular geographic region. Furthermore, the reliance on the honeybee for insecticide toxicity assessments may compromise more susceptible pollinator species, such as stingless bees, and thus impair agricultural production and plant diversity in the neotropics. [4,13,14]

The general focus on the impact of neonicotinoids on pollinators, particularly honeybees, has led to an expansion and incentives of reduced-risk pesticides and particularly of biopesticides. [15-18] The

encouragement for the use of such compounds is illustrated by European Pesticide Regulation No. 1107/2009/EC and Directive 2009/128/EC of the European Parliament and of the Council in addition to similar regulatory efforts in Canada, the USA, and elsewhere. [17,19,20] Nonetheless, reduced-risk insecticides may still be highly toxic and represent a high risk to non-target beneficial insects such as stingless bees, which are completely neglected in ecotoxicology and risk-assessment studies. Furthermore, biopesticides are not necessarily safer than synthetic pesticides, because origin is not a determinant of toxicity or risk. [21-24]

Considering the shortcomings regarding toxicological and ecotoxicological assessments on non-*Apis* bee species, such stingless bees, and reduced-risk (bio)insecticides as presented above, here we hypothesized that the oral and contact (acute) toxicity of the recommended label rates of a reduced-risk insecticide (chlorantraniliprole), a bioinsecticide (azadirachtin), and a reduced-risk bioinsecticide (spinosad) might compromise the survival of two species of stingless bees, *Partamona helleri* (Friese) and *Scaptotrigona xanthotrica* (Moure) (Hymenoptera: Apidae: Meliponini). Such stingless bees' species are important native pollinators in the Neotropical America. [10-13,25] The group activity and flight take-off of adult workers exposed to azadirachtin or chlorantraniliprole were also assessed for impact prediction on behavior of both native bee species.

2. Materials and methods

2.1 Insects and insecticides

Three colonies of each of the stingless bee species *P. helleri* (ca. 1,000-3,000 individuals/colony) and *S. xanthotrica* (over 10,000 individuals/colony) were collected in Viçosa county (State of Minas Gerais, Brazil; 20° 45′ S and 42° 52′ W) and maintained in the experimental apiary of the Federal University of Viçosa. The adult workers of each species were collected as groups of 10 individuals per colony at the hive entrance of their respective colonies in the experimental apiary using glass jars when they exit the hive to forage. They were subsequently taken to the laboratory and maintained without food inside wooden cages covered with organza (35 x 35 x 35 cm) for 1 h at 25 ± 2°C, 70 ± 10% RH, and total darkness until the bioassays were initiated. The waiting period before exposure was necessary to standardize the feeding condition of the tested workers.

Three insecticides were used in their respective commercial formulations as follows: azadirachtin (emulsifiable concentrate at 12 g litre^{-1}, DVA Agro Brasil, Campinas, SP, Brazil), chlorantraniliprole (suspension concentrate at 200 g litre^{-1}, DuPont do Brasil, Barueri, SP, Brazil), and spinosad (suspension concentrate at 480 g litre^{-1}, Dow AgroSciences, Santo Amaro, SP, Brazil). The neonicotinoid imidacloprid (water dispersible granules at 700 g kg^{-1}, Bayer CropScience, São Paulo, SP, Brazil) was used as a positive control due to its high and widely recognized toxicity to bee pollinators. [5,6,26] The insecticides were used at rates calculated based on the spray volume per hectare (azadirachtin: 1000 l ha^{-1}, chlorantraniliprole: 1000 l ha^{-1}, spinosad: 400 l ha^{-1}, imidacloprid: 333 l ha^{-1}) for the control of the white fly *Bemisia tabaci* (Gennadius) (Hemiptera: Sternorrhyncha: Aleyrodidae) and the tomato pinworm *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) on tomato crops in accordance with the recommendations of the Brazilian Ministry of Agriculture. [26] The insecticide formulations were diluted either in distilled and deionized water (contact exposure bioassays) or in an aqueous sucrose solution 500 g kg^{-1} (for oral exposure bioassays) at the following concentrations based on the maximum field label rates registered for each insecticide: azadirachtin at 30 mg litre^{-1}, chlorantraniliprole at 3 mg litre^{-1}, imidacloprid at 42 mg litre^{-1}, and spinosad at 20.4 mg litre^{-1}. [26]

2.2 Time-mortality residual contact bioassays

Inner walls of transparent low-density polyethylene plastic containers (volume of 250 mL and inner surface of 365.43 cm^2) with negligible sorption and resistant to organic chemicals under short-term exposure [27,28] were treated with 500 µl of insecticide solution (or water, in the case of

the control) using an artist's air brush (Sagyma SW440A, Yamar Brasil, São Paulo, SP, Brazil) coupled with an air pump (Prismatec 131A Tipo 2 VC, Itu, SP, Brazil) at a pressure of 6.9 x 10^4 Pa. The insecticide-sprayed containers were allowed to dry for 2 h under a fume hood at 25 ± 3°C without incidence of direct light, after which 10 adult workers were released within each container and retained by covering the top with organza fabric. Three containers (replicates), one per colony of each species, were used. Untreated sucrose solution was provided in a feeder to the bees through a hole in the plastic containers. After a 3-h exposure, the insects were transferred to untreated containers with 1 mL of sucrose solution at 500 g kg^{-1}. Bee survival was recorded hourly for 24 h from the beginning of the residual contact exposure. The insects were considered dead when they were unable to walk the length of their body and no insect recognized as dead by such criteria was able to recover in the study.

2.3 Time-mortality ingestion bioassays

Low-density plastic containers (250 ml) were again used as experimental units containing 10 worker bees fed on 500 µl of insecticide-contaminated sucrose solution (except for untreated controls) in longitudinally cut Eppendorf tubes used as plastic feeders and inserted through a hole in the plastic container. The insecticide dose ingested was obtained by weighing the feeders before and after the experiment. The oral ingestion of insecticide-contaminated sucrose solution (500 mg kg^{-1}) by each 1-h starved bee species (between 0.69 and 1.12 µl adult $worker^{-1}$ of *P. helleri,* and between 0.52 and 0.77 µl adult $worker^{-1}$ of *S. xanthotrica*) led to the following ingested doses of insecticide per worker: *P. helleri* - 25.80 ng bee of azadirachtin, 2.84 ng bee^{-1} of chlorantraniliprole, 28.90 ng bee^{-1} imidacloprid, and 22.79 ng bee^{-1} of spinosad*;* and *S. xanthotrica* - 15.48 ng bee^{-1} of azadirachtin, 2.06 ng bee^{-1} of chlorantraniliprole, 25.28 ng bee^{-1} imidacloprid, and 15.82 ng bee^{-1} of spinosad. Three containers (replicates), one per colony of each species, were used. Bee survival was recorded as previously described for the contact bioassays.

2.4 Group activity

Bioassays of the overall group activity of workers of both stingless bee species were performed 24 h after the period of exposure (contact and ingestion) to azadirachtin and chlorantraniliprole, in addition to the distilled water-treated control. Imidacloprid and spinosad were not used in the sublethal (behavior) bioassays, due to 100% mortality by both contact and oral exposure obtained with the field label rates of these insecticides. The insects were exposed either by contact or ingestion, as previously described, and subsequently transferred to glass Petri dishes (9.0 cm diameter) in groups of 10 workers bees from the same colony and three different colonies (i.e., replicates) of each species. The bottom of each Petri dish was covered with filter paper (Whatman no. 1), and the dish was covered with transparent plastic film to prevent insect escape. Activity recording was performed after a 1 h acclimation to the Petri dish arena to prevent confounding effects derived from insect handling. The overall insect activity was recorded for 10 min and digitally transferred to a video-tracking system equipped with a digital CCD camera (ViewPoint LifeSciences, Montreal, QC, Canada). The overall insect activity was recorded as changes in pixels between two subsequent pictures of the insect group, which were registered every 10^{-2} s. The changes of quantified pixels between the subsequent pictures represented all movements within the arena (including walking, body part movements, and conspecific interactions) that were captured by the system every 10^{-2} s. The bioassays were performed at 25 ± 2°C and under artificial fluorescent light between 2:00 and 6:00 p.m.

2.2 Flight take-off bioassay

The workers subjected to the group activity bioassays were subsequently subjected to flight take-off bioassays 25 h after the period of exposure. [29] The same number of workers was used per replicate (i.e., 10) in three replicates (i.e., colonies) per treatment. A 105 cm tall tower was formed with three stacked wooden cages (35 x 35 x 35 cm each) opened in their interior to allow free

insect flight through them. A fluorescent lamp was placed 15 cm above the top of the tower in a dark room. The flight take-off bioassay explored the vertical bee flight towards the light source after the insect release from the center bottom of the tower. The flight take-off was recorded within 1 min of worker release and was designated as follows: I) no flight (i.e., bee remained on the base of the tower), II) flight up to 35 cm high, III) flight between 36 and 70 cm high, IV) flight between 71 and 105 cm high, and V) flight reaching the light source at a height of 120 cm.

2.3 Statistical analyses

The data from the time-mortality (survival) bioassays were subjected to survival analyses using Kaplan-Meier estimators to obtain the survival curves and estimates of the median survival time (LT_{50}) (PROC LIFETEST in SAS). [30] The insects still alive at the end of the bioassays were treated as censored data. The overall similarity among survival curves (and estimated LT_{50}s) was tested by the χ^2 Log-Rank test, and the pairwise comparisons between curves were tested using the Bonferroni method. The data from the overall group activity were subjected to analyses of variance after being checked for normality and homoscedasticity (PROC UNIVARIATE from SAS) [30], which were satisfied. The results of flight take-off were subjected to the (non-parametric) Kruskal-Wallis test ($p < 0.05$) (PROC NPAR1WAY from SAS). [30]

3. Results3.1 Time-mortality by contact exposure

The survival of *P. helleri* and *S. xanthotrica* after insecticide contact exposure exhibited a significant difference among the treatments (*P. helleri*: Log-rank $\chi^2 = 229.42$, df = 4, $p < 0.001$; *S. xanthotrica*: Log-rank $\chi^2 = 215.57$, df = 4, $p < 0.001$) (Fig. 1(A,C)). Azadirachtin and chlorantraniliprole did not cause any mortality within 24 h among adult workers of *P. helleri*, resembling the untreated control (with only water application), but imidacloprid and spinosad caused 100% mortality within 5 h with median lethal times ($LT_{50} \pm SE$) of 0.25 ± 0.00 h and 1.00 ± 0.14 h, respectively (Fig. 1B). A similar trend was also observed for *S. xanthotrica* with azadirachtin and chlorantraniliprole exhibiting negligible mortality with 24 h exposure, and imidacloprid and spinosad leading to 100% mortality within 5 h of exposure ($LT_{50} \pm SE$ of 0.25 ± 0.00 h for imidacloprid and 4.00 ± 0.00 h for spinosad) (Fig. 1D). LT_{50}'s for azadirachtin, chlorantraniliprole and untreated control were not shown because the mortality did not exceed 50%, which is the minimum value that need to be reached throughout the time for estimation of such parameter.

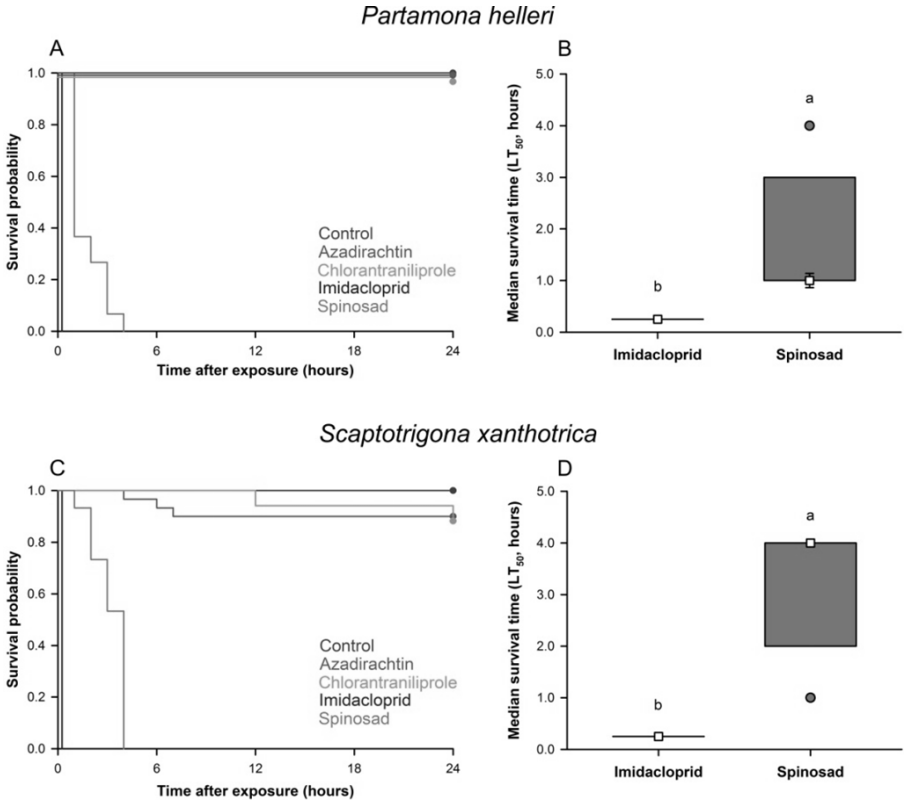

Figure 1 Survival curves (**A, C**) and box plots of the median survival times (LT_{50}'s) (**B, D**) of workers of the Neotropical stingless bee species *Partamona helleri* (**A, B**) and *Scaptotrigona xanthotrica* (**C, D**) contact-exposed to the field rates of commercial insecticides. *Box plots* indicate the median (line within the box), mean (open square with standard error bars) and range of dispersion (lower and upper quartiles, represented as the limits of the box, and outliers (symbol)) of the LT_{50}s. The box plots with different lower case letters are significantly different by Bonferroni's method ($p < 0.05$).

3.2 Time-mortality by oral exposure

The survival curves of adult workers exposed to the insecticides by ingestion also exhibited trends similar to those obtained by contact exposure. The insecticides led to significant differences in the mortality profile of both *P. helleri* (Log-rank $\chi^2 = 189.24$, df = 4, $p < 0.001$) and *S. xanthotrica* (Log-rank $\chi^2 = 209.60$, df = 4, $p < 0.001$) (Fig. 2(A,C)). Azadirachtin and chlorantraniliprole led to negligible mortality for both stingless bee species, once again resembling the control. In contrast, imidacloprid and spinosad led quickly to 100% mortality of adult workers of *P. helleri* (LT_{50}'s ± SE of 0.25 ± 0.03 h for imidacloprid and 2.00 ± 0.00 h for spinosad) (Fig. 2B) and *S. xanthotrica* (LT_{50}'s ± SE of 0.25 ± 0.00 h for imidacloprid and 2.00 ± 0.00 h for spinosad) (Fig. 2D).

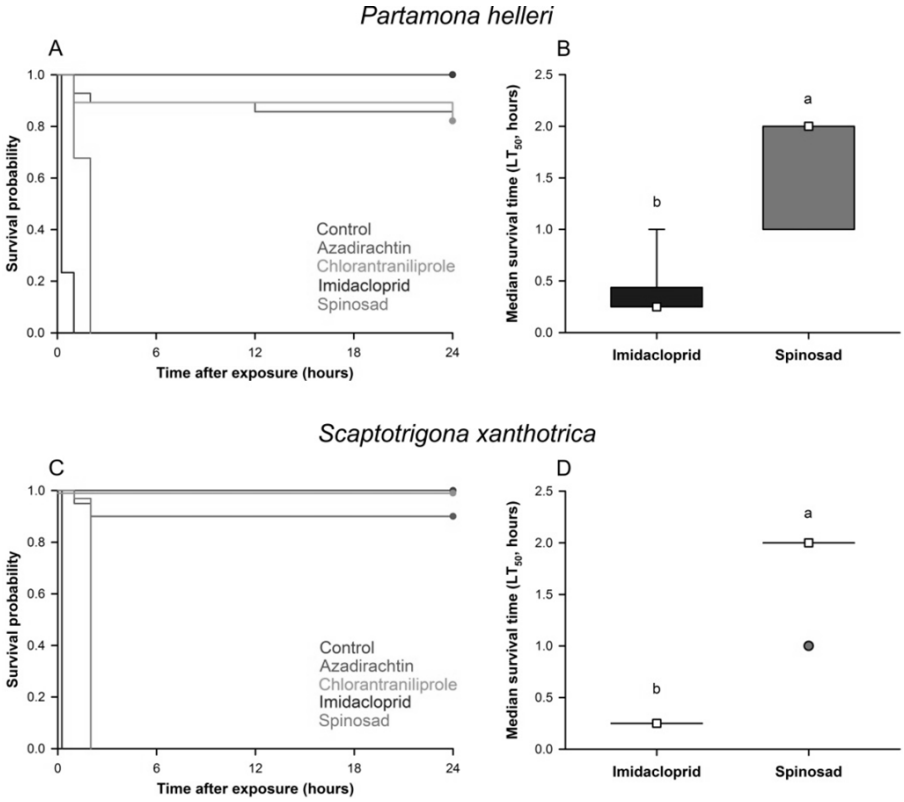

Figure 2 Survival curves (**A, C**) and box plots of the median survival times (LT_{50}'s) (**B, D**) of workers of the Neotropical stingless bee species *Partamona helleri* (**A, B**) and *Scaptotrigona xanthotrica* (**C, D**) orally-exposed to the field rates of commercial insecticides. *Box plots* indicate the median (line within the box), mean (open square with standard error bars) and range of dispersion (lower and upper quartiles, represented as the limits of the box, and outliers (symbol)) of the LT_{50}s. The box plots with different lower case letters are significantly different by Bonferroni's method ($p < 0.05$).

3.3 Overall group activity

The group activity was assessed for azadirachtin- and chlorantraniliprole-exposed insects and unexposed insects (control), but no significant effect was detected ($F_{2,7} < 1.45$ $p > 0.31$). The mean overall activity (± SE) was 46.70 ± 13.56 Δ pixels/s x 10^{-2} and 66.98 ± 16.76 Δ pixels/s x 10^{-2} for *P. helleri* among the treatments with contact and oral exposure, respectively, and 206.01 ± 31.80 Δ pixels/s x 10^{-2} and 302.35 ± 23.33 Δ pixels/s x 10^{-2} for *S. xanthotrica* among the treatments with contact and oral exposure, respectively.

3.4 Flight take-off activity

Contact exposure to azadirachtin did not affect the take-off flight of *P. helleri* (H = 0.40, df = 1, p = 0.53) (Fig. 3A), whreas chlorantraniliprole significantly impaired such flight preventing bees from reaching the light source (H = 4.50, df = 1, p = 0.03) (Fig. 3B). In contrast, both insecticides impaired flight take-off of *S. xanthotrica* (H > 13.40, df = 1, $p < 0.001$) (Fig. 3(C,D)).

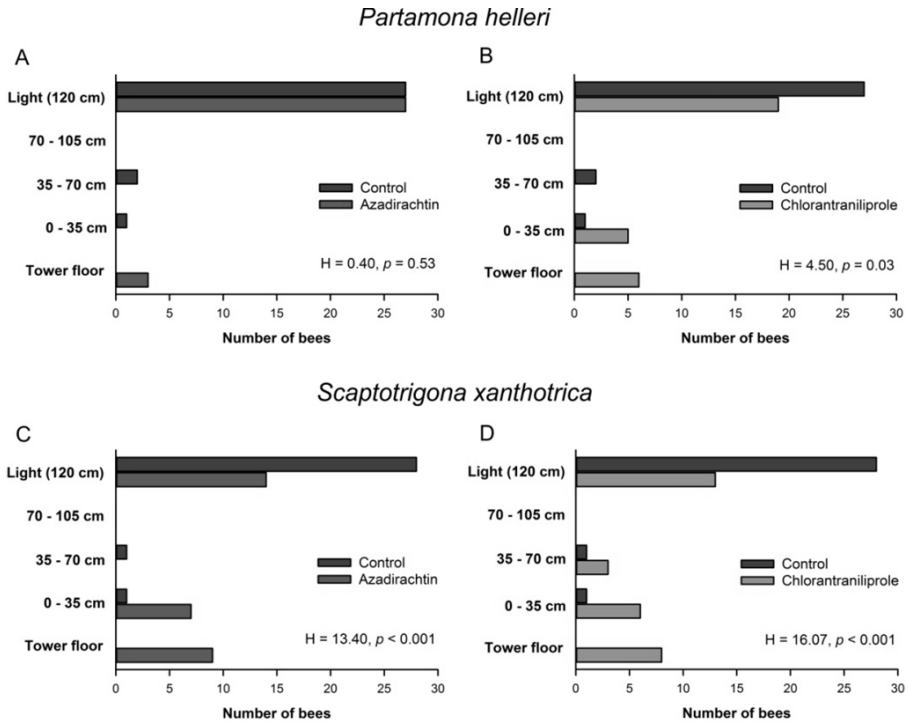

Figure 3 Flight take-off activity of adult workers of the Neotropical stingless bee species *Partamona helleri* (**A, B**) and *Scaptotrigona xanthotrica* (**C, D**) contact-exposed to the field rates of the commercial insecticides azadirachtin (**A, C**) and chlorantraniliprole (**B, D**). The results of the (non-parametric) Kruskal-Wallis test ($p < 0.05$) used to test the differences between untreated and insecticide-treated insects are indicated.

Oral ingestion of either azadirachtin or chlorantraniliprole impaired flight take-off by *P. helleri* ($H > 4.98$, $df = 1$, $p \leq 0.02$), reducing the number of individuals taking-off for flight and the number reaching the light source (Fig. 4(A,B)). By contrast, there was no significant effect of azadirachtin and chlorantraniliprole on *S. xanthotrica* regarding their flight take-off activity ($H \leq 1.16$, $df = 1$, $p \geq 0.28$) (Fig. 4(C,D)).

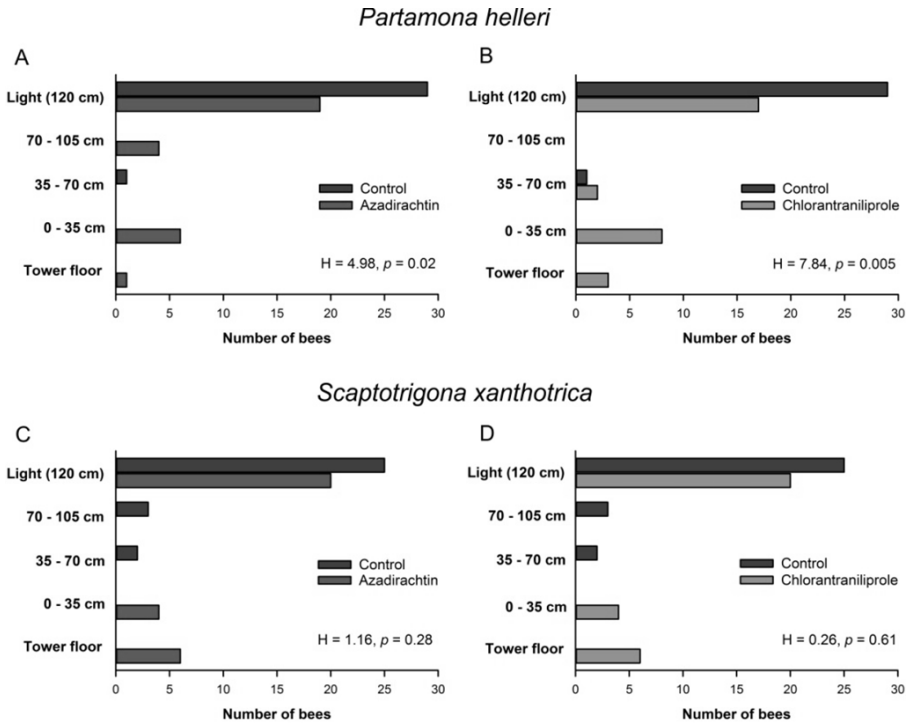

Figure 4 Flight take-off activity of adult workers of the Neotropical stingless bee species *Partamona helleri* (**A, B**) and *Scaptotrigona xanthotrica* (**C, D**) orally-exposed to the field rates of the commercial insecticides azadirachtin (**A, C**) and chlorantraniliprole (**B, D**). The results of the (non-parametric) Kruskal-Wallis test ($p < 0.05$) used to test the differences between untreated and insecticide-treated insects are indicated.

4. DiscussionThe susceptibility of stingless bees to modern substances defined as reduced-risk insecticides, including bioinsecticides, has received little attention. Here we observed that spinosad is highly toxic at 20.4 mg litre^{-1} to both stingless bee species tested, *P. helleri* and *S. xanthotrica*, causing quick and complete mortality of the worker bees within 5 h of either contact or oral exposure. Only imidacloprid exhibited more rapid mortality of workers than spinosad, regardless of the exposure method.

The terpenoid bioinsecticide azadirachtin caused negligible adult mortality in both species of stingless bees used in this study, similar to the reduced-risk diamide insecticide chlorantraniliprole. The low acute mortality caused by azadirachtin and chlorantraniliprole was expected, because the former usually requires very high doses to achieve repellence and impair development in Hymenoptera, [31] and the latter exhibits insecticidal activity limited to caterpillars, flies and beetles, [32,33] with low toxicity against honeybees and bumblebees at the recommended field label rate. [34,35] The differential ryanodine receptor sensitivity to chlorantraniliprole in bee pollinators is the likely reason for the low acute toxicity of this insecticide to bee species, [33,36] whereas the reasons for the low azadirachtin acute toxicity to pollinators have not yet been studied.

As sublethal exposure may also compromise insect survival and reproduction of bees, the sublethal responses of *P. helleri* and *S. xanthotrica* to azadirachtin and chlorantraniliprole were also assessed. Here, azadirachtin and chlorantraniliprole did not affect overall group activity of workers, which is an important trait since represents insect-insect interactions and individual activity within a group of social bees. However, flight take-off of *P. helleri* was impaired by chlorantraniliprole, and the flight take-off of *S. xanthotrica* was impaired by azadirachtin and chlorantraniliprole, regardless of the route of exposure. Neither compound has been reported to impair pollinator activity, unlike

neonicotinoids in honeybees, [37,38] and neonicotinoids and pyrethroids in bumblebees. [39,40] However, azadirachtin and chlorantraniliprole have not been subjected to such studies, which is likely due to their perceived (although questionable) overall environmental safety. Nonetheless, the azadirachtin interference with the availability of brain neurosecretory peptides and the chlorantraniliprole interference with muscle activity may allow for the flight take-off impairment. [31,32]

Our findings partially support the perceived notion of the environmental safety of azadirachtin and chlorantraniliprole at their recommended field rates in a worst case scenario, which is reinforced by their recognition as reduced-risk insecticides (or bioinsecticide, in the case of azadirachtin). However, such a perception is not valid for spinosad, another reduced-risk (bio)insecticide, which exhibited high acute lethality to the two stingless bee species tested, resembling the drastic and broadly recognized toxicity of imidacloprid to pollinators. [41-44] Furthermore, azadirachtin and chlorantraniliprole impaired the flight take-off of stingless bees, potentially impairing foraging and compromising colony survival, as may happened with honeybees under sublethal impact of neonicotinoids. [36,45] Therefore, the perceived notion of pollinator safety associated with reduced-risk insecticides is misleading; low toxicity to non-target species is only one of the alternative requirements (which are fairly broad) allowing the recognition of a given insecticide as a reduced-risk compound. [16] Regarding bioinsecticides, origin is not a determinant of toxicity, and the perceived safety of such compounds is again a misconception. The proper assessment of such compounds should not be neglected by being labeled as reduced-risk insecticides and/or as bioinsecticides before a proper assessment has been performed.

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